Chapter 1 INTRODUCTION

The advent of antibiotics is a breakthrough in the healthcare sector that has helped in saving millions of lives and reducing the incidences of various infectious diseases. However, the concrete advancement in control of microbial infections needs to combat the parallel upsurge in drug resistance. Antibiotic resistance has caused an alarming situation in today's world. The armory of antimicrobials is becoming less effective leaving very few reliable options. The healthcare market is intimidated by the infrastructure and costs of introducing an entirely new drug in the market. The feasibility and reliability of developing new antimicrobial molecules are being questioned. Methicillin-resistant *Staphylococcus aureus* (MRSA) has rendered many conventional antibiotics obsolete. MRSA leads to deactivation and inefficacy of antibiotics. The irrational/overuse use of vancomycin and linezolid against Methicillin-resistant *Staphylococcus aureus* (MRSA) has given rise to many of the resistant isolates. Among the new generation novel antimicrobials, lipopeptide antibiotics have appeared as stars of the current times. Daptomycin is a capable member of the lipopeptide antibiotic group which has exhibited a broad spectrum of activity against antibiotic-resistant species of *Staphylococcus aureus* and *Enterococci*. This antibiotic does not surrender to the mechanisms of resistance as in case of beta-lactam antibiotics, vancomycin, linezolid etc. for gram-positive pathogens. The most distinctive mechanism of action and minimal drug resistance profile along with strong antimicrobial characteristics make Daptomycin promising anti-MRSA alternative.

1.1 Introduction to Daptomycin

Antibiotic resistance has become a serious clinical issue in today's world. The failure of generations of antibiotics to combat "Superbugs" has threatened the healthcare sector. Though several antimicrobials are being worked upon still there is an uncertainty regarding their efficacy and affordability. Methicillin-resistant *Staphylococcus aureus* (MRSA) has crippled several established antibiotics [Stefani *et al*., 2003]. The overuse of popular antibiotic against drug-resistant species has resulted in resistant isolates. The smart antibiotic-resistant strains have averted vancomycin penetration through thickening of the cell walls [Cui *et al*., 2003]. There is a necessity for potential antibiotics against MRSA activity with a modified mechanism of action [Judith *et al*., 2005]. Lipopeptide antibiotics are novel antibiotics against MRSA with a unique mechanism of action [Dohmen *et al*., 2013]. They are produced *via* non-ribosomal peptide biosynthetic mechanism. Such antibiotics have an acyl group linked to linear or cyclic peptide chain which may be cationic or anionic residues [Robbel and Marheil *et al*., 2010].

Lipopeptide antibiotics as shown in Figure 1.1, range from cyclic or short linear peptides attached to lipid tail or certain lipophilic molecule(s). Their applications range in various spheres of industrial arena including the pharmaceuticals as antibiotics, cosmetics as anti-wrinkle agents and emulsifiers in food processing industries; and also in biotechnology as biosurfactants. Lipopeptides, such as daptomycin, caspofungin, micafungin, anidulafungin and A54145, CDA4, and Friulimicin have attained commercial antibiotic status [Pirri *et al*., 2009].

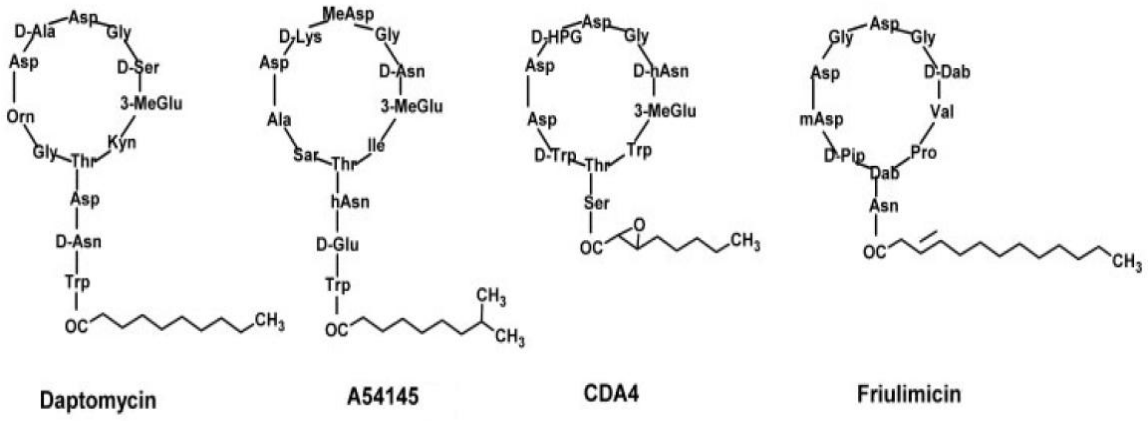

Figure 1.1 Members of lipopeptide antibiotic family [Shi *et al*., 2012]

Daptomycin is a potential antibiotic belonging to the lipopeptide antibiotic family, which can tackle pathogenic effect of wide spectra of gram-positive bacteria, like Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci. Its peculiar antimicrobial mechanism engages in calcium reliant loss of membrane potential ceasing cellular functions [Silverman, 2003].

1.2 The Challenge of Methicillin-Resistant Microorganisms

Methicillin-resistant microorganisms have posed threat to the life-saving drugs for a long time. The advent of β-lactam resistance in 1944 came as a serious challenge [Gots, 1945]. Methicillin came into existence in 1956 and after two years, Methicillin-resistant *Staphylococcus aureus* (MRSA) was found [Smith *et al*., 1962]. Since then, drug-resistant species have become widespread. Studies suggest the prevalence of 59% of resistant isolates in case of skin infections in the US [Moran *et al*., 2006]. In France, 3.6%, while in Greece-75% of MRSA strains were reported [Dauwalder *et al*., 2008, Chini *et al*.,2006].The popular tricyclic glycopeptides, Vancomycin produced by *Streptomyces orientalis* was discovered in the year 1956. It has been a popular drug for a long time in drug-resistant issues.

Vancomycin has side-effects, like nephrotoxicity and ototoxicity [Sivagnanam and Deleu, 2003]. In 1996 the first vancomycin resistance was revealed. The thickening of the bacterial cell wall has hampered the efficacy of vancomycin [Tenover *et al*., 1998]. In order to combat such problems, proper drug monitoring is required [Steinkraus, 2007]. Linezolid is another valuable drug, the first of w oxazolidinone class against antibiotic susceptible as well as antibiotic-resistant aerobic gram-positive bacteria. Though Linezolid can be orally administered it gives rise to few health problems such as bone marrow depression and health risks to patients with solid organ transplantation. In this context, there is a need for a drug that is effective on MRSA and has minimal side-effects [Kerry and Hartmut,2010].

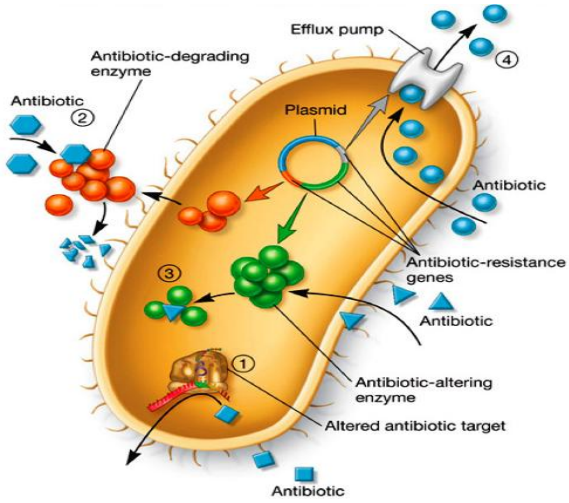

Figure 1.2 Mechanisms of drug resistance in MRSA [Todar, 2014.]

Resistance in MRSA is dictated by multiple factors as shown in Figure 1.2, mecA gene which instructs the unique penicillin-binding PBP-2a protein can impair 4 high-binding-affinity PBPs when exposed to antibiotics. Pathogenic growth proliferates at the infected sites because of the low affinity of PBP-2a towards methicillin. Genes mecR1 and mecI control and regulate methicillin-resistant phenotype and lead to PBP-2a. β lactamase and

penicillinases make MRSA resistant to β lactams and penicillins. Aminoglycoside modulating enzymes in cases like gentamicin, tobramycin, etc. have prompted drug resistance. Synthesis of D-Ala-D-Lac (peptidoglycan precursors) in place of D-Ala-D-Ala cause vancomycin and teicoplanin resistance. The efflux pump machinery of cells poses resistance to certain antimicrobials like tetracyclines or fluoroquinolones. Vancomycin is a potent antibiotic for the treatment of MRSA. MRSA have recently caused failures of vancomycin therapy in case of bacteremia and fluoroquinolones. Therefore, alternative antibiotic compounds against MRSA (Table 1.1) are being worked and pondered upon which include drugs like linezolid, daptomycin, and ceftaroline. These anti-MRSA antibiotics must be considered for treating bacteremia, endocarditis, or MRSA pneumonia. Owing to such issues, Daptomycin has received much notice as a potential anti-infective drug [Blair *et al*., 2015; Dvorchik *et al*.,2003].

Table 1.1 A comparative study of Daptomycin with other popular anti-MRSA drugs [Dvorchik *et al*., 2003].

Properties	**Vancomycin**	**Daptomycin**	**Linezolid**
Class of antibiotics	Glycopeptide	Lipopeptide	Oxazolidinone
Mode of action	Inhibits cell-wall synthesis	Calcium-dependent dissipation of membrane potential	Inhibits protein synthesis
Applications	Severe infections caused by susceptible strains of methicillin-	Complicated skin and skin structure infections *S.aureus,*	Complicated skin and skin structure infections including

	resistant *S.aureus*. As a combinatorial drug with aminoglycoside for endocarditis	blood stream infections (bacteremia),including those with right-sided infective endocarditis, caused by MRSA isolates	diabetic foot ulcers caused by MSSA. Treats Vancomycin resistant *E.faecium* infections
Bioavailibility	Incomplete absorption	Complete absorption	Incomplete absorption
Clearance	0.06 L/h/kg	0.10 L/h/kg	0.01 L/h/kg
Volume of Distribution	0.3 to 0.43 L/kg	0.7-0.8 L/kg	0.1 L/kg
Half Life	4-6 h	4-5 h	8h
Protein Binding	55%	31%	93%
Tissue penetration and effects	Average	High	Low
Dosing therapy	1 gm q12h	4 mg/kg IV qd	600 mgq 12h

1.3 Discovery of Daptomycin

The journey of Daptomycin started in the early 1990s. This cyclic anionic 13-amino acid lipopeptide is the first official antibiotic of A21978C family. It is a secondary metabolite of *Streptomyces roseosporus* fermentation process. It was initially developed at Eli Lilly and Company, using decanoic acid as a precursor, but was held over due to problems like

myopathy. In 1997, Cubist Pharmaceuticals Inc. took over the worldwide rights from Eli Lilly and Company and continued the research [Dohmen et al., 2013]. Daptomycin has a hydrophilic inside with a decanoyl side chain as a lipophilic tail. The formula of Daptomycin is C72H101N17O26; the molecular weight is 1620.67, while its empirical name is N-decanoyl-L tryptophyl-D-asparaginyl-L-aspartyl-L-threonylglycyl-L-ornithyl-L-aspartyl-D-alanyl-Laspartylglycyl-D-seryl-three-3-methyl-L-glutamyl-3-anthraniloyl-L-alanine_1-lactone as shown in Figure 1.3 [Dvorchik *et al*.,2003].The first trials were done at a dose of 4 mg two times a day but owing to several cases of high serum levels of creatine kinase (CK) causing skeletal muscle toxicity, the trials were stopped[Dvorchik *et al*.,2003]. In 1999, clinical trials were resumed and further concrete research led to the FDA approval of Daptomycin for skin and skin structure infection at a dosage of 4 mg/kg daily in the year 2003. FDA authorization was granted for the cure of bloodstream infections and right-sided endocarditis due to Methicillin susceptible *Staphylococcus aureus* as well as Methicillin resistant *Staphylococcus aureus* in 2006 [Tally and De Bruin, 2000].

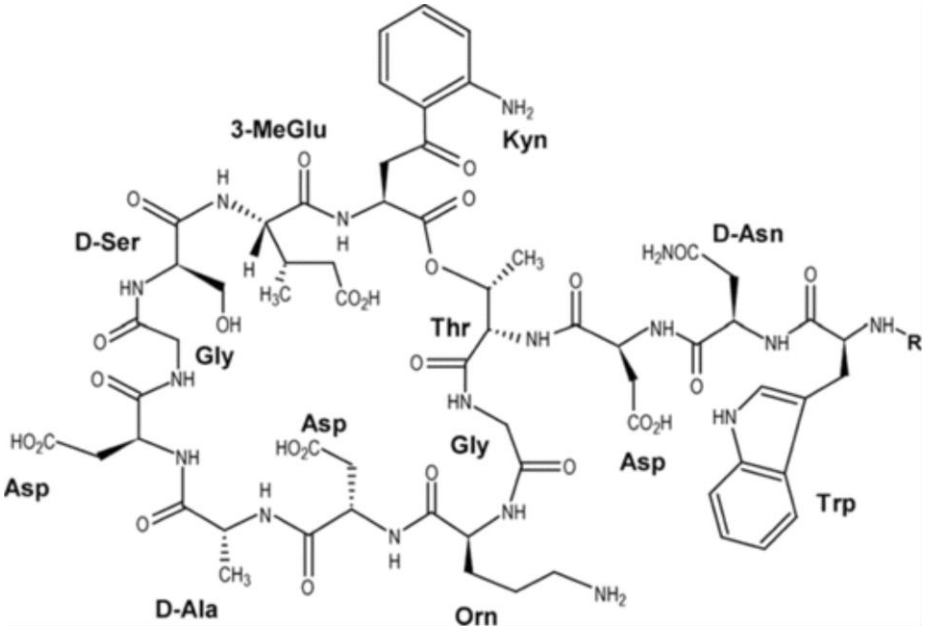

Fig. 1.3 Chemical Structure of Daptomycin [Einstein *et al*., 2010**].**

1.4 Development of Daptomycin

The mechanism of biological synthesis of Daptomycin as shown in Figure 1.4 has always remained an inquisitive and interesting phenomenon. Various possible strategies have been applied to increase the production and activity of this useful antibiotic. Literature suggests that Daptomycin has a peculiar structure consisting of 13 amino acids and shares a ten-membered macrolactone ring and three exocyclic residues. The fatty acyl-moiety is linked to the *N*-terminal Trp1. The cyclic peptide core consists the assembly of a set of nonproteinogenic amino acids including D-Asn2, Orn6, D-Ala8, D-Ser11, (2*S*, 3R)-methyl glutamate (MeGlu) and kynurenine (Kyn13), that gives rise to the ester bond with Thr4 and forms the macrolactone ring[Miao *et al*.,2005]. Daptomycin contains a specific EF-hand motif (DXDG) found in the ribosomally assembled calmodulin which helps in $Ca2^{+}$ binding for antimicrobial activity [Yazawa and Yagi,1980]. D-configured amino acids form the cyclic core to which the long chain fatty acid is attached. The conventional biosynthesis of Daptomycin is dictated by DptA, DptBC and DptD, three nonribosomal peptide synthetases (NRPS), and *in trans*-acting enzymes in *S. roseosporus* [Fischbach and Walsh, 2006].

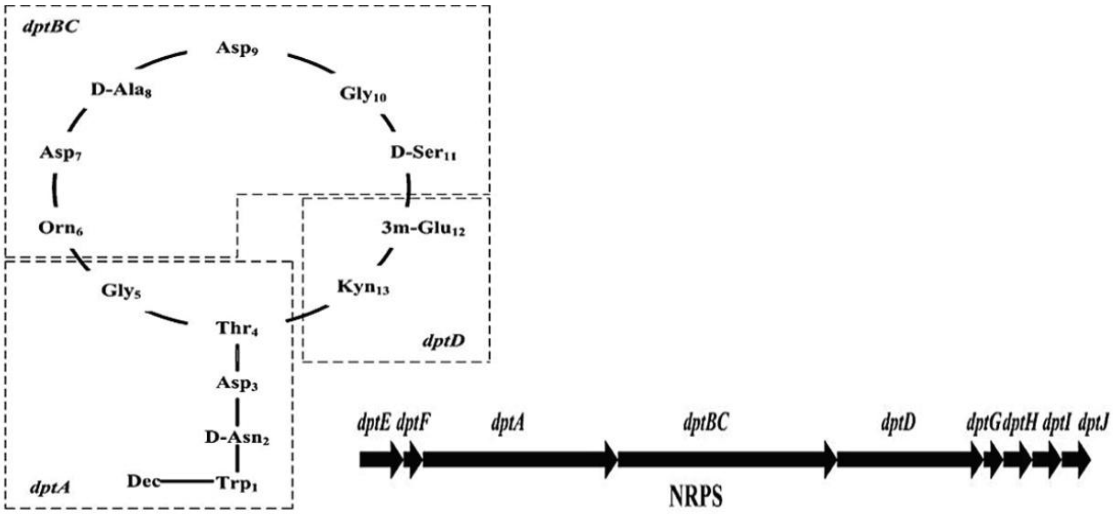

Fig. 1.4 Representation of the Biosynthesis of Daptomycin [Tally and De Bruin, 2000]

1.4.1 The multidomain organization of Nonribosomal peptide synthetases (NRPS)

Nonribosomal peptide synthesis involves nonribosomal peptide-synthetase (NRPS) enzymes without engaging the messenger RNA. Each module plays a unique role in the systematic incorporation of the building blocks into the long oligopeptide chain and can be further divided into catalytic domains as shown in Figure 1.5 [Fischbach and Walsh, 2006]. The responsible genes for Daptomycin production found in *S. roseosporus* has 12 known genes clustered in a region spanning about 100 kb. The entire peptide chain is by a nonribosomal peptide synthetase (NRPS) mechanism and containing three genes encoding NRPS (dptA, dptBC, and dptD), two genes for activating the fatty acid (dptE and dptF), seven genes for precursor supply, resistance and transport (dptG, dptH, dptI, dptJ, dptM, dptN, and dptP) [Coeffet-Le Gal *et al*., 2006], with a co-regulation modality.

1.4.1.1 N-terminal ACYLATION

Acylation of the N-terminal amino acid in Daptomycin is essential for its unique mechanism of action. DptE and DptF account for activation of fatty acid which is then catalyzed by N-

terminal C domain of DptA to form N-acylation of Trp1. DptE, a novel acyl-AMP ligase, activates fatty acid with ATP consumption, then transfers fatty acids from acyl AMP to the 40-phosphopantetheine group of DptF which is an acyl carrier protein (ACP) [Wittmann *et al*., 2008].

1.4.1.2 BIOSYNTHESIS OF NONPROTEINOGENIC AMINO ACIDS

13 amino acids form the core of Daptomycin. 7 L-amino acids are accepted and activated by cognate A domain and included in the core peptide and 3D-amino acids (D-Asn2, D-Ala8, D-ser11) are integrated into the L-form and associated E-domains in DptA, DptBC govern the stereochemical changes. Kyn13 and MeGlu12 forming genes were recognized in Daptomycin structure, only Orn was a product of primary metabolism to conduct Daptomycin biosynthesis. Kynurenine is a product of the kynurenine pathway from degradation of L-tryptophan. The resultant of Tryptophan degradation by tryptophan-2, 3-dioxygenase (TDO) is N-formylkynurenine.Finally, the removal of formyl group by N-formylkynurenine formamidase forms kynurenine [Kurnasov *et al*., 2003]. Consequently, kynurenine is generated NAD in eukaryotes controlling metabolic activities and biological synthesis of few rare secondary metabolites [Lima *et al*., 2009; Sheoran *et al*., 2008; Matthijs *et al*., 2004]. dptJ encoded recognized tryptophan-2, 3-dioxygenase (TDO) is the rate-limiting enzyme which can affect Daptomycin yield upto 50% [Nguyen *et al*., 2006].Tryptophan-2, 3-dioxygenase (TDO) encoding gene inside chromosome SSGG3688 encoding TDO and SSGG3689 encoding kynureninease help in kynurenine generating anthranilate. It is customary that isozymes (e.g., SSGG3688 and DptJ) present in the gene structure and one more locus in the chromosome to regulate antibiotic production. MeGlu is

important for daptomycin biosynthesis. dptI encodes a SAM-dependent methyltransferase. Removal of dptGHIJ gives rise to formation of Daptomycin replacing Glu12 with MeGlu12 [Nguyen *et al*., 2006]. The balance of the final mutant with dptJ resumes the Daptomycin production containing MeGlu12, indicating that dptI helps in methylation of Glu. At present, biochemical analysis showed that dptI encodes a novel methyltransferase adding a methyl group from SAM to ketoglutarate to form 3-methyl-2-oxoglutarate, which after transamination yields MeGlu [Mahlert *et al*., 2007].

1.4.1.3 PEPTIDE FORMATION AND RELEASE

DptA, DptBC, and DptD are engaged in peptide formation followed by the release of Daptomycin. DptA has five modules which bring together first five amino acids. At the N-terminal of DptA, a special C-domain (type CIII) couples the long chain fatty acids to the N-terminal of Trp1. E-domain of module 2 epimerizes the L-Asn2 to give D-Asn2. DptBC assembles another six amino acids. E-domain in module 8 and module 11 are responsible for generating D-Ala8 and D-ser11, respectively. DptD containing two modules is responsible for forming MeGlu and Kyn, and a thioesterase domain catalyzes the creation of ester bond between Kyn13 and Thr4 and releases the completed Daptomycin from the NRPS multienzyme [Robbel and Marheil,2010].

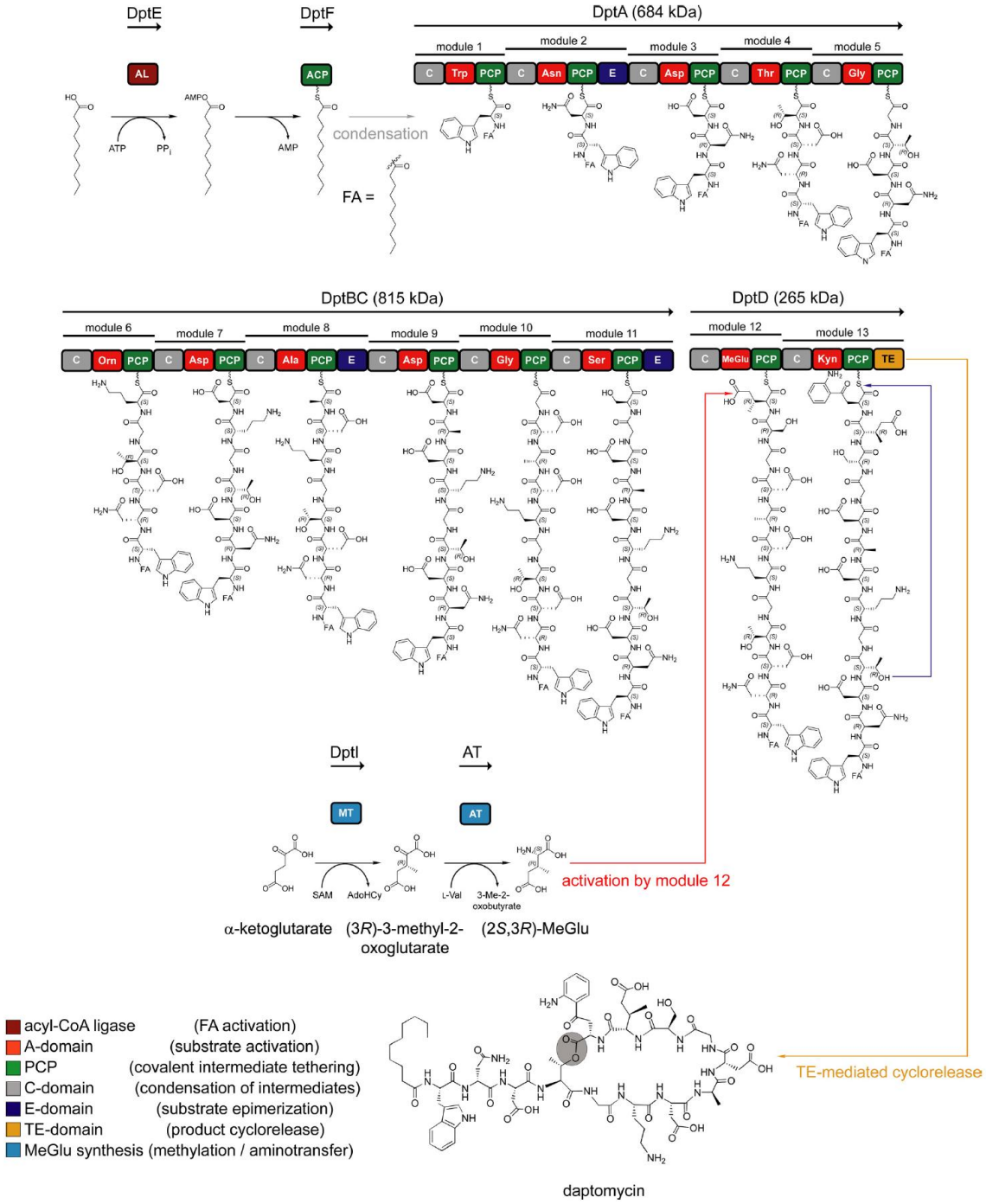

Figure 1.5 Schematic overview of Daptomycin biosynthesis by *S. roseosporus* [Robbel and Marheil,2010].

The assembly of the peptide chain is directed by mainly three NRPSs DptA, DptBC, and DptD, consisting 43 catalytic domains. The commencement of Daptomycin biosynthesis is governed by DptE and DptF which activate and include the FA moiety. The N-terminal CIII-domain of DptA control the transfer of the DptF-bound FA onto the amino group of Trp1. The synthesis of MeGlu12 is carried out by the *S*-adenosyl methionine (*SAM*)-dependent methyltransferase DptI and a presently not known aminotransferase. Cyclorelease is carried out by the C-terminal TE domain of DptD.

1.4.2 Combinatorial Approach for Biosynthesis

The combinatorial advancement for biosynthesis of Daptomycin has received attention as it constitutes of the entire reprogramming of genes regulating the enzymatic machinery of the antibiotic [Walsh, 2002]. Genetic manipulations of *S. roseosporus* can be achieved by gene cluster sequencing, cloning and heterologously expression [Penn *et al*., 2006].

Hybrid biosynthesis has been extensively carried out for *S. roseosporus* [Debono *et al*., 1988; Baltz *et al*., 2006]. The modular NRPS assembly line proposes substitutions and alterations via designing and development from a single module to multiple modules. At first, *dptA* and *dptD* were removed from the original locus [Coeffet-Le Gal *et al*., 2006]. These genes were further introduced into *S. roseosporus* to trans-complement deletions of *dptA* and *dptD* by the formation of plasmid cloning vectors leading to conjugal transfer of genetic data from *Escherichia coli* to the producer strain [Mc Henney and Baltz,1996].

1.5 Mode of action of Daptomycin

Daptomycin, unlike its counterparts, has a stronger mechanism to deal with MRSA. The popular antibiotic Linezolid acts by inhibition of the protein synthesis in pathogenic cells

hence disrupting translation of messenger RNA (mRNA) into proteins in the ribosome. This antibiotic binds to the 23S portion of the 50S subunit [Ament *et al*., 2002] and prevents initiation complex formation. Most Gram-negative bacteria involve the role of efflux pumps which eject linezolid out of the cell much before it could act. Vancomycin prevents overall cell wall formation in gram-positive bacteria (Schumacher *et al*., 2006). Its large hydrophilic molecule can form hydrogen bond interactions with the terminal D-Ala-D-Ala moieties of the NAM/NAG-peptides to prevent synthesis of N-acetylmuramic acid (NAM) and N-acetyl glucosamine (NAG) polymers which form the main backbone of the cell wall. But, in resistant bacteria, the last D-ala residue gets substituted by a D-lactate, so vancomycin fails to bind which otherwise bound to the peptide chains does not allow the interaction with the cell wall cross-linking enzyme transpeptidase. The distinctive mode of action of Daptomycin is depicted in Figure 1.6. The early studies by Jung *et al*. projected two-step activity derived from NMR, CD and Fluorescence Spectroscopy profiles for structural alterations. At first, Ca2+ attaches to Daptomycin in solution and generate a conformational change, escalating amphipathic nature and lowering the charge [Jung *et al*., 2004]. The oligomerization begins and tends to micelle formation which leads to Daptomycin interaction with cell membranes. Then, in the second step, Ca2+ link Daptomycin and the acidic phospholipids. Daptomycin after structural transition gets deeper insertion into the bilayer membrane as the CD measurements suggest.

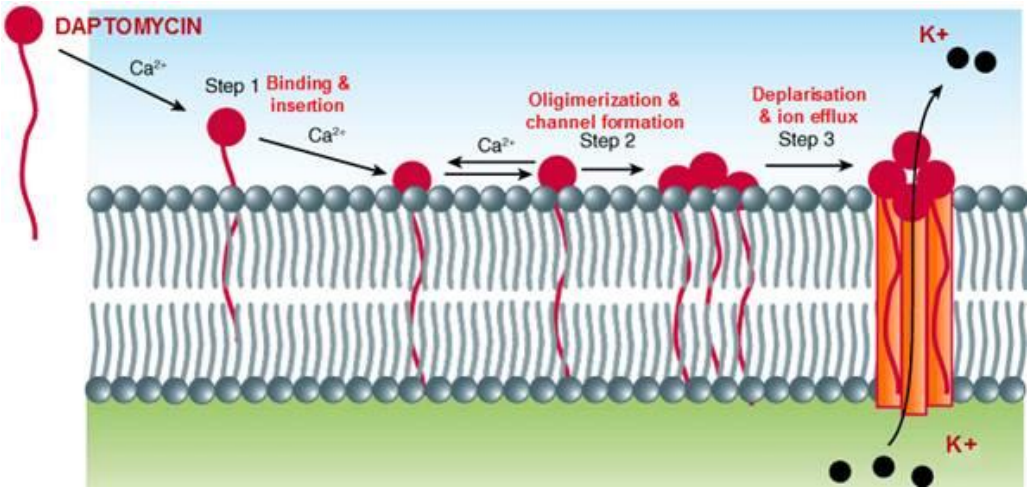

Figure 1.6 The mode of action of Daptomycin [Jung *et al*., 2004].

There have been few contradictions as studied by Silverman *et al*. Studies proposed that cytoplasmic membrane depolarization was not the only reason for cell death as it occurred consequently[Silverman *et al*., 2003]. The recent studies found that Mg2+ also supports micelle formation, but no conformational change is seen. Daptomycin formed oligomers with 14-16 monomers Ca2+ was added in a 1:1-ratio. Jung *et al*. proposed a phenomenon wherein the divalent cations cover the negatively charged residues and lead micelle formation through stacking interactions between aromatic residues or through the arrangement of the lipid tails towards the inner side of the micelle [Jung *et al*., 2004]. Scott *et al*., 2007 validate that Daptomycin faces a minor conformational rearrangement when binds to DHPC in the presence of Ca2+.

1.6 Clinical Applications of Daptomycin-Case Studies

1.6.1 Wound infections post cardiac surgery

Medical studies have reported the occurrence of deep sternal wound infection post cardiac surgery, *Staphylococcus aureus* being the most common isolate from affected wound

sternotomies. This infection caused a higher rate of morbidity and mortality. The observational survey involved 23 cases of post-surgery deep sternal wound infection with gram-positive bacteria in February 2009 and September 2010. The prevalence of deep sternal wound infection was 1.46%. The mean dose of Daptomycin application was 4.4 ± 0.9 mg/kg/d and the average duration of the Daptomycin application was 14.47 ± 7.33 days. Medication of deep sternal wound infection caused by gram-positive bacteria with Daptomycin was found to be efficient in healing wound [Popov *et al*.,2011].

1.6.2 Diabetic foot ulcers and infections

Out of the 103 patients infected with diabetic foot ulcer caused by *Staphylococcus aureus*, 47 were given Daptomycin dosage while 56 were given a comparator. The clinical and microbiological efficiency and safety of Daptomycin were close to the popular antimicrobials for curing infected diabetic foot ulcers. [Lipsky and Stoutenburgh,2005].

1.6.3 Combination drugs containing Daptomycin for Vancomycin-Resistant *Enterococcus faecium*

Fosfomycin can effectively cure vancomycin-resistant enterococcus (VRE) infections. 32 urinary stent infected patients after renal transplant surgery were vulnerable to linezolid, fosfomycin, and daptomycin but resistant to amoxicillin, nitrofurantoin, minocycline, based on their MIC50s and MIC90s. The best results could be obtained using the combination of fosfomycin and daptomycin (2.8 to 3.9log10 CFU/ml kill; $P<0.001$) or amoxicillin (2.6 to 3.4; $P<0.05$) which could treat the urinary infections with proficiency [Descourourez *et al*., 2013].

1.6.4 Daptomycin for osteomyelitis caused by MRSA in a renal transplant recipient with Fabry Anderson disease

Daptomycin has proven its benefits for adults in the treatment of bone and skin infections by MRSA. It was used to treat Fabry-Anderson Disease with right heel osteomyelitis. Teicoplanin and Tigecycline failed to cure the problem whereas Daptomycin alone at 4 mg/Kg/qd for 4 weeks could effectively heal Osteomyelitis [Polilli *et al*., 2012].

1.6.5 Brain Damage and Hearing Loss in Infant Rat Pneumococcal Meningitis

Increased cerebrospinal fluid inflammation as an effect of damage by β-lactam antibiotics lead to neurological issues in bacterial meningitis. Daptomycin otherwise prevents such inflammation as well as brain injury as compared to β-lactam like ceftriaxone. In case of clinical use for pediatric bacterial meningitis, synergistic daptomycin or rifampin with ceftriaxone was injected in infant rat pneumococcal meningitis model. Cerebrospinal fluid was taken at 6 and 22 h after the initiation of treatment and was evaluated for concentrations of defined chemokines and cytokines. Brain damage and hearing loss were assessed by histomorphometry. Daptomycin along with ceftriaxone versus ceftriaxone considerably (P<0.04) lowered Cerebrospinal fluid concentrations of monocyte chemoattractant protein 1 (MCP-1), MIP-1_, and interleukin 6 (IL-6) at 6 h and MIP-1_, IL-6, and IL-10 at 22 h after start of therapy, led to sufficiently (P<0.01) less apoptosis, and considerable (P<0.01) cure of hearing issue. Rifampin and ceftriaxone versus ceftriaxone also led to reduced Cerebrospinal fluid inflammation (P<0.02 for IL-6 at 6 h), no effect was shown on apoptosis and hearing problem. Combinatorial daptomycin medication could show further benefits for the cure of pediatric pneumococcal meningitis [Denis *et al*., 2012].

1.6.6 Immunomodulatory effects of Daptomycin.

Daptomycin demonstrated immunomodulatory properties by the suppression of role of cytokine after immunogenic stimulation by MRSA. Research studies indicated the tremendous impact of the combinatorial effect of the Daptomycin with vitamin E in treating wounds by MRSA, especially after cardiac surgery. [Theodor,2014].

1.6.7 Prosthetic joint infection by *Enterococcus faecalis*

Enterococci are associated with 2.3% of prosthetic joint infections. Such issue poses economic challenges on health care systems. To deal post-operation conditions of infections, Daptomycin proves to be an effective medical boon. This clinical case gave an evidence of prolonged use this crucial antibiotic. [Rafael *et al*., 2010].